IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF OKLAHOMA

STATE OF OKLAHOMA,	)	
	)	
Plaintiff,	)	
	)	
v.	)	**Case No. 05-cv-329-GKF(PJC)**
	)	
TYSON FOODS, INC., et al.,	)	
	)	
Defendants.	)	

DECLARATION OF VALERIE J. HARWOOD, Ph.D.

I, Valerie J. Harwood, Ph.D., hereby declare as follows:

1. My terminal degree is a Ph.D. in Biomedical Sciences from Old Dominion University & Eastern Virginia Medical School in Norfolk, VA (1992). From 1992 to 1995 I held a full-time postdoctoral research position at the University of Maryland Center of Marine Biotechnology. In 1995 I joined the Department of Natural Sciences at the University of North Florida as a tenure-track Assistant Professor, where I taught microbiology and related courses, and maintained a research laboratory until I joined the University of South Florida (USF) in Tampa, FL in August 1998. Since that time I have been employed by USF in the Department of Biology (now the Department of Integrative Biology) in a full-time position. In 2004 I was promoted to Associate Professor, which is my current rank, and was awarded tenure. My responsibilities at USF include teaching undergraduate and graduate courses in microbiology, mentoring undergraduate and graduate research students, and maintaining an active research program. My research laboratory personnel currently include one technician and six Ph.D. students. My research focuses on microbial water quality, with particular emphasis on microbial source tracking (MST), a field of environmental microbiology that seeks to determine the source of fecal contamination in water by identifying specific molecular signatures in the DNA of fecal microorganisms.

2. I have worked in the field of environmental microbiology since 1986, and in the area of MST since 1997. I am the author of 34 peer-reviewed journal articles and

three peer-reviewed, published reports, twelve of which are directly related to MST. One of these articles has been cited in other peer-reviewed publications 121 times to date (100 citations is an important benchmark that few papers reach). Other publications include over 30 technical reports, a book chapter, and substantial contributions to the U.S. Environmental Protection Agency Microbial Source Tracking Guide Document. I am also co-editor of a book on microbial source tracking that is contracted to be published by Springer Scientific Press in 2010, and I have been an invited speaker on water quality research and MST over 50 times across the U.S., in the U.K. and in New Zealand. I am a reviewer for many scientific journals including Environmental Science & Technology, Environmental Microbiology, and Journal of Applied Microbiology, and am a member of the editorial review board of Applied & Environmental Microbiology. I have served on state and federal grant panels including Sea Grant, National Oceanic and Atmospheric Administration (NOAA) and the United States Department of Agriculture (USDA), and have been awarded over $3 million in grant funding from various agencies including the National Science Foundation, NOAA, Sea Grant, USDA, United States Environmental Protection Agency (USEPA) and National Institutes of Health. My current funding for MST and related environmental microbiology research totals over one million dollars from agencies including the Florida Department of Environmental Protection, the Water Environment Research Foundation, the US Department of Agriculture and the US Environmental Protection Agency.

3. I have studied the Daubert Motion of the defendants in the case that is before the court. The major points I will make in this document are as follow:

- My opinion that the spreading of fecal-contaminated poultry litter on fields in the Illinois River watershed (IRW) represents a threat to human health was formed prior to the development and use of the poultry litter biomarker assay and was not based solely, or even substantially, on this test. Rather, my opinion was based on the weight of evidence represented by data from published literature, land use, the amount of litter applied to fields, the pathogens that are known to be shed in poultry feces, environmental sampling and analysis, and the hydrology and geology of the IRW. The poultry litter biomarker is one tool among many used to

build the weight of evidence that land application of poultry litter represents a substantial danger to human health.

- There is a difference between scientific *theory* and the methodology surrounding that theory and the application of that methodology to a specific target. The theory and basic concepts entailed in using polymerase chain reaction (PCR) to detect a specific gene that belongs to a specific bacterium that is specific to a certain animal (or to humans) has a decade-long history in the scientific literature (Bernhard and Field 2000; Field and Samadpour, 2007; Stoeckel and Harwood, 2007). Furthermore, the use of PCR as a diagnostic and forensic tool has an even longer history, dating back to the late 1980s. The scientific theory behind the use of PCR to very specifically detect bacterial strains and to discriminate them from closely-related strains is not novel, and has a long history in the scientific literature. We have applied the accepted and demonstrated theory of PCR-based detection of specific bacteria in the environment to a particular target, the poultry litter biomarker.

- From the time my Expert Report was produced until the present, substantial additions to our knowledge about the poultry litter biomarker have been made. The poultry litter biomarker work has been peer-reviewed and has now also been reproduced in blind trials by an independent researcher (Dr. Michael Sadowsky, University of Minnesota). Although the manuscript was rejected by Applied and Environmental Microbiology (AEM), this is not an unusual fate for high-quality scientific work. In fact, AEM rejects about 65% of the manuscripts it receives. Our practice as scientists is to consider the reviewers' criticisms (some of which are generally valid and some of which are not) and revise the manuscript accordingly, then to resubmit it to the same or another journal. In this case, the main reason for rejection of the manuscript in its revised form was lack of geographic representation (samples from across the country were not tested, which limits the widespread applicability of the method). We have responded to these criticisms and have tested samples from a wider geographic range.

A detailed discussion of the above points is presented below.

I. The spreading of fecal-contaminated poultry litter on fields in the Illinois River watershed (IRW) represents a threat to human health.

4. My expert report details the many lines of evidence that I relied on to form my expert opinion. Most of these lines of evidence are independent of the poultry litter biomarker data. Section II of my expert report presents the basis of my opinion on the possible sources of waterborne disease in the IRW; a synopsis is presented here.

5. One of the most common routes of disease transmission is the waterborne route, in which people ingest, inhale or encounter water that contains microbial pathogens. Many waterborne pathogens enter water primarily via fecal material from humans and animals. Enteric disease that is transmitted by the waterborne route is greatly underreported (reviewed in (Leclerc, Schwartzbrod & Dei-Cas, 2002), leading to an underestimate of the economic and public health impact of specific diseases, including salmonellosis and campylobacteriosis (Allos, 2001) (Mead et al., 1999) (Voetsch et al., 2004). As summarized by the World Health Organization (WHO):

> "Infections and illness due to recreational water contact are generally mild and so difficult to detect through routine surveillance systems. Even where illness is more severe, it may still be difficult to attribute to water exposure. Targeted epidemiological studies, however, have shown a number of adverse health outcomes (including gastrointestinal and respiratory infections) to be associated with faecally polluted recreational water. This can result in a significant burden of disease and economic loss." (WHO, 2003).

6. Many diseases, called zoonoses, are spread from animals that harbor human pathogens in their gastrointestinal tract. Campylobacteriosis and salmonellosis are important zoonoses in the U.S. (DuPont, 2007; Leclerc et al., 2002), and are transmitted in water contaminated by poultry feces and those of other animals (Leclerc et al., 2002; National Research Council, 2004). *Escherichia coli* strains that are pathogenic to humans, such as *E. coli* O157:H7 (Dipineto et al., 2006; Doane et al., 2007; Doyle & Schoeni, 1987) have been isolated from poultry. The 2007 U.S. EPA Report of the Experts Scientific Workshop on Critical Research Needs for the Development of New or Revised Recreational Water Quality Criteria recognized the importance of zoonoses to human health risk, and placed the highest priority for further research on contamination from poultry and other agricultural animals (U.S. Environmental Protection Agency, 2007).

7. Individuals differ in their susceptibility to infection by waterborne pathogens (Belanger & Shryock, 2007; U.S. Environmental Protection Agency, 2005a; World Health Organization, 2003). Infants and children, elderly and immunocompromised individuals have less robust immune systems than others, and are thus more susceptible to infection and more likely to suffer severe outcomes from an infection (Leclerc et al., 2002). Young children have less developed immune systems than adults and are likely to swallow more water and are thus more likely to become ill than healthy adults (World Health Organization, 2003). A very recent study showed that the largest risk factor for gastroenteritis in children caused by *Campylobacter, Salmonella* or pathogenic *E. coli* strains was recreational water use, rather than consumption of certain foods (Denno et al., 2009).

8. Section III of my expert report details the relationship between fecal indicator bacteria (e.g. enterococci and *E. coli*) and human health risk with respect to recreational water use. The general reliability and practicality of protecting public health by enumerating fecal indicator bacteria has led to the continued use of this practice worldwide for over 100 years. The U.S. EPA has focused their previous epidemiological studies on water bodies contaminated with human sewage, and developed the standards on that basis (U.S. Environmental Protection Agency, 1986) ***Because contamination of waters by other sources could be just as detrimental to human health, the EPA water quality standards apply to all recreational waters regardless of the source of contamination.*** The very low infectious dose of certain poultry-associated pathogens such as *Campylobacter jejuni* and *Salmonella* (Leclerc et al., 2002; Skovgaard, 2007; U.S. Environmental Protection Agency, 2005a) is among the reasons that the U.S. EPA is very concerned about the impact of poultry waste on water quality and human health (U.S. Environmental Protection Agency 2005a, 2007).

9. Recreational water quality standards based on indicator bacteria concentrations have been supported in multiple studies since EPA's 1986 water quality criteria were published, e.g. (Pruss, 1998) (Wade et al., 2003). Both the WHO (World Health Organization, 2003) and the European Union (EU) have adopted standards for recreational water quality that are based on indicator bacteria concentrations (enterococci and/or *E. coli*). In 2007 the U.S. EPA convened a group of experts to consider the impact

of recent scientific and technical advances on recreational water quality criteria and implementation of standards (U.S. Environmental Protection Agency, 2007). The report recommended updating the criteria by incorporating new methods (e.g. quantitative PCR) and continuing to perform epidemiology studies in areas affected by various pollution sources, but also recommended the continuation of the current practice of enumerating indicator bacteria. Thus, indicator bacteria standards will doubtless be used to protect the health of recreational water users in the U.S. in the foreseeable future.

10. Based on these lines of evidence, my expert opinion was and is that (1) the disease burden from waterborne sources is significantly underreported and constitutes an important economic and human health impact in the U.S.; (2) zoonoses such as campylobacteriosis and salmonellosis are transmitted by waterborne routes and are significantly underreported; (3) poultry feces and contaminated litter are known to contain these human pathogens and are thus high-risk sources of fecal contamination and indicator bacteria to water bodies; (4) indicator bacteria are useful surrogates for pathogens in waters known to be impacted by high-risk sources of fecal contamination.

11. Section IV of my Expert Report discusses water quality and the characteristics of the IRW. Indicator bacteria levels in many of the IRW tributaries routinely exceed Oklahoma water quality standards, therefore these water bodies have been placed on the State's 303(d) list of impaired waters. The IRW contains many miles of small tributaries, providing proximity of water bodies to fields on which poultry litter has been applied. The Illinois River, which is an Oklahoma Scenic River, is considered to be too polluted by fecal bacteria to support its designated use of primary body contact recreation. Dr. Teaf's Expert Report for this case describes the extent of impairment in the IRW; in summary over 75% of the Illinois River and its major tributaries are listed as impaired by high bacteria levels (Teaf, 2008). Enterococci are responsible for many of the water quality exceedances throughout the IRW (Teaf, 2008). This group of fecal indicator bacteria is recognized as measure of recreational water quality by the U.S. EPA and the State of Oklahoma, and its levels are correlated with the risk of gastroenteritis in recreational water users in fresh and salt water (U.S. Environmental Protection Agency 1986; Wade et al 2003).

12. Many water samples that were collected by experts for the State. Some of these samples were analyzed more than 6 hours after collection due to the necessity for shipping samples to a lab on the west coast. These samples were always shipped on ice, and arrived cold at the analytical laboratory. The 6 hour holding time required by the U.S. EPA and U.S. Geological Survey (USGS) are stipulated for samples that are taken for regulatory purposes, e.g. beach water quality monitoring or assessment of ambient water quality for TMDL programs. The stipulation is made because bacteria tend to die off in samples that are held for long periods. Thus, the effect of extended holding time is that bacterial concentrations will be *lower* than if the analysis was done immediately. Most studies, however, have found that no significant differences result when samples are held 24-48 hours at 8 - 10° C (refrigerated or on ice). For example, the study on which U.S. EPA and USGS regulations are based found that 21.5% of samples that were tested for fecal coliforms after 24 hours of refrigeration decreased in concentration, while only 3.5% of samples showed an increased concentration (The Public Health Laboratory Service Water Sub-Committee, 1953). The majority of samples (75%) showed no change. Because fewer samples showed a change when held for 6 hours compared to 24 hours (the only two times tested), the authors recommended the 6 hour holding time. In the words of the authors:

> "There is a much greater probability (1 in 20) of a large change, particularly in the direction of a decrease, in either the coliform or faecal *coli* content of a sample if it is stored for 24 hr at either room or refrigerator temperature before the test is set up."

Other studies have corroborated these findings; water samples held at refrigerator temperatures for 24 or up to 48 hours experience no change, or a decrease in bacterial concentrations (Pope et al., 2003; Selvakumar et al., 2004). Standard Methods for the Examination of Water and Wastewater stipulates that ambient water samples collected for non-regulatory purposes can be held for 24 hours (Sec 9060B) (American Public Health Association, 2005).

13. An analysis of indicator bacteria (fecal coliform) loading from various potential sources in the six counties that contribute to the IRW was conducted for this investigation (Teaf, 2008). Pets, deer and wildlife, and human sources (i.e. septic systems, wastewater treatment plant discharges) together accounted for an estimated

1.4% of total loading of fecal coliforms to the IRW, while livestock accounted for 98.6%. Poultry and cattle contributed an approximately equal, major load (estimated at 41% and 44% of all livestock contributions, respectively). Contaminated poultry litter and soil receiving land-applied poultry litter contains an even higher load of enterococci than fecal coliforms; thus poultry are doubtless a dominant source of fecal indicator bacteria to the IRW.

14. Below the surface layer of soil in the IRW is a karst substratum that is riddled with cracks and fissures (Fisher, 2008). The effect of this karst terrain is that surface water and groundwater have a much greater physical connection than they do in other geological formations, and contaminants from the surface, including bacteria, can readily penetrate the shallow aquifer, and from there can find their way to deeper aquifers such as those used for drinking water (Davis, Hamilton & Van Brahana, 2005). Evidence for the widespread influence of surface contamination on groundwater quality in the IRW is that indicator bacteria were isolated from springs, shallow wells and deep wells (detailed in Teaf, 2008). Almost 1700 wells are registered for drinking water use in the Oklahoma portion of the IRW (Fisher, 2008). The owners of these wells generally do not disinfect or otherwise treat the water from the wells, therefore people can be exposed to pathogens that infiltrate the groundwater via runoff.

15. Management of poultry litter/manure in the IRW is by land application, which is considered a passive waste management approach that can impact surface and ground water quality as microorganisms move with surface and subsurface water flow (U.S. Environmental Protection Agency, 2005a). The U.S. Department of Agriculture has also found that land application of poultry waste can result in extensive runoff of bacteria to surface waters (Sistani et al., 2009). The State of Oklahoma recognizes the potential impact of poultry operations and other agriculture on water quality. Under the Oklahoma Registered Poultry Feeding Operation Act, it is required that "...there shall be no discharge to waters of the state." (Title 2; Registered Poultry Feeding Operation Act). Broiler production generates 340 tons of contaminated litter annually from a farm with only four houses (Dozier, Lacy & Vest, 2001), and used poultry litter is known to contain high levels of indicator bacteria and also contains pathogens such as *Campylobacter* and *Salmonella* (Berndtson et al 1992; Santos et al 2005). According to Dr. Bert Fisher's

Expert Report, from 2000-2007 over one billion birds (chickens and turkeys) were produced by the defendants in the IRW (Fisher, 2008), representing an average of over 141 million bird/year. In 2005-2006 there were over 1,900 active poultry houses in the IRW, generating an estimated 354,000 tons of waste (Fisher, 2008). This material is spread on fields, generally within three to five miles of the area where it was produced, where it can leach into groundwater and run off into surface water (Fisher, 2008).

16. Contaminated poultry litter samples were collected by CDM from poultry houses in the IRW in 2006 (Camp Dresser & McKee (CDM), 2008). Ten samples, each from a different facility, were tested for indicator bacteria level, which proved to be exceedingly high. The geometric mean was ~1200 *E. coli* per gram of litter, and ~51,000 enterococci/g litter, with maximum levels over 100,000/g litter (Camp Dresser & McKee (CDM), 2008). *Salmonella* was detected in four of 24 contaminated poultry litter samples (16.7%), but *Campylobacter* was not detected by the culture-based methods used. More sensitive PCR methods that could detect stressed (viable but nonculturable) pathogens would have been more suited to the detection of *Salmonella* and *Campylobacter* in poultry litter and environmental samples; however, they were not utilized in this study. Given the near-ubiquitous association of these pathogens with poultry feces, my opinion is that these pathogens were present, but that too few were present in a culturable state to be detected by the methods used, which were developed for the food industry and not for environmental samples where pathogens are typically physiologically stressed.

17. Traditional fate and transport studies are unusual in the microbiology field because of the potential hazards involved in releasing pathogenic microorganisms into the environment, and the difficulty associated with extrapolating data derived from laboratory experiments to environmental scenarios. In this study, the hypothetical pathway of surface water contamination from land-applied poultry litter was followed from litter, to soils that had received land application of litter, to runoff from the edges of fields. "Edge-of-field" samples collected by CDM in the IRW typically had very high levels of indicator bacteria (Camp Dresser & McKee (CDM), 2008). Some samples had *E. coli* levels of over 1 million/100 ml, which approaches the concentration found in raw sewage (Harwood et al., 2005). Soil samples collected from fields on which poultry litter had been land-applied as levels of up to 2,000 *E. coli* per gram of soil and 17,000

enterococci/g. As expected, IRW surface water samples had variable indicator bacteria levels; however, chronic exceedances of the primary body contact standard for bacteria levels were recorded throughout the IRW (detailed in Teaf, 2008). Indicator bacteria were also found in groundwater, indicating transport of the microorganisms from surface to subsurface waters.

18. Dr. Roger Olsen analyzed an array of chemical and bacterial parameters using the multivariate statistical method of principle components analysis, which revealed a distinctive "signature" that is characteristic of soils and waters contaminated by poultry waste (Olsen, 2008). The measured parameters included metals, nutrients, physical measurements and indicator bacteria. A definitive poultry waste signature was derived from phosphorus, bacteria, organic carbon, potassium, copper, zinc, and nitrogen-containing compounds. This signature was found in all sample types throughout the IRW, including edge-of-field, soils impacted by land application, rivers, streams, and their sediments, groundwater, and Lake Tenkiller. Olsen concluded that a significant source of bacterial contamination in the IRW was poultry waste, and that the signature was present at every leg of the transport pathway from litter to soil to edge-of-field samples to surface water and ground water (Olsen, 2008).

19. Based on these lines of evidence, my expert opinion is that (1) Although the area is largely rural, the surface waters of the IRW contain elevated indicator bacteria levels that demonstrate a health risk to recreational water users; (2) the great number of surface water tributaries and the karst subsurface pre-dispose IRW surface and ground waters to contamination from surface influences; (3) used poultry litter is disposed of by land application in the IRW, which is known to have a high potential for contaminating water bodies; (4) the poultry litter contains high bacterial and nutrient levels; (5) bacterial loading from poultry feces is one of the two dominant sources in the IRW; (6) human exposure to waters contaminated by poultry litter occurs and constitutes a risk to human health.

II. The theory that PCR can very specifically detect and quantify bacteria is not novel, nor is the methodology of developing a PCR-based test for a bacterium that is specific to a particular host (the animal or human that harbors the bacterium).

20. In science, a theory is a well-accepted explanation of a particular class of observations. A theory follows a coherent analytical structure and is well-supported by data. The theory behind the use of PCR to detect specific DNA sequences is grounded in the biochemistry of DNA synthesis, which is common to all living things. Enzymes called DNA polymerases have an astoundingly faithful mechanism for copying DNA strands, and these enzymes require a very small string of DNA bases (subunits) to start their task. We can duplicate a very specific DNA sequence by PCR by capitalizing on the nature of DNA bases; they pair very specifically with a complementary base (like a lock and key); therefore we can design short pieces of DNA called "primers" to reliably target a specific DNA sequence and provide a starting point for the DNA polymerase. We design a pair of primers because DNA is made up of two non-identical "strands"; one primer targets each strand for duplication. We then use a DNA polymerase (enzyme) to begin the synthesis of this specific fragment of DNA. The PCR process repeatedly cycles the steps of primer binding, elongation of each newly synthesized DNA fragment and melting of the product to allow a new round of synthesis, so that a very small amount of target DNA can serve as a template for the synthesis of a large enough quantity of DNA to detect and further analyze.

21. Because the amount of target DNA required for amplification is so small, many applications of PCR do not require culture of the organisms that bear the target sequence. This ability to carry out culture-independent analysis of samples has revolutionized the clinical (Murakawa et al., 1988), forensic (Higuchi et al., 1988) and environmental microbiology fields over the past twenty years.

22. The hypothesis that polymerase chain reaction would be useful for detecting a specific bacterial DNA sequence in an environmental sample was first tested in 1988, soon after the development of PCR (Steffan & Atlas, 1988). The success of the early experiments resulted in a burgeoning array of applications of the PCR methodology for addressing questions in environmental microbiology. Prior to this development, in 1985, *Bacteroides fragilis* had been suggested as a human-specific marker of fecal

contamination (Allsop & Stickler, 1985). This work was followed a decade later by the publication of a paper suggesting the use of PCR to detect host-associated *Bacteroides* species in feces and in water (Kreader, 1995). The development of new PCR-based MST methods increased after the publication of the first paper that did not rely on any cultured organisms to develop PCR primers for host-specific targets (Bernhard & Field, 2000). We have essentially followed the method of Bernhard and Field to develop the poultry litter biomarker assay. This methodology is grounded in two-decade old theory of the PCR, and in a similar duration of hypothesis testing for MST (Kaspar et al., 1990; Krumperman, 1983; Stoeckel & Harwood, 2007).

23. MST methods can be roughly grouped into library-dependent and library-independent approaches. **Library-dependent** methods typically begin by culturing, or growing, indicator bacteria such as *E. coli* or enterococci from the feces or sewage of various host species (e.g. chickens, cattle, humans) that may impact water quality in the study area. The isolates are typed, or "fingerprinted" by highly discriminatory laboratory methods, and their fingerprints make up the known source library. Fingerprinting can be carried out by a variety of phenotypic methods, including antibiotic resistance analysis (Hagedorn et al., 1999; Harwood, Whitlock & Withington, 2000; Wiggins, 1996) and carbon source utilization (Harwood et al., 2003). Genotypic fingerprinting, which detects differences among strains at the genetic level, can also be carried out by a number of methods, including ribotyping (Moore et al., 2005; Parveen et al., 1999), pulsed field gel electrophoresis (Stoeckel et al., 2004), and rep-PCR (Johnson et al., 2004).

24. MST libraries are expensive and time-consuming to construct, and their applications across geographical distance or over time spans over one year has not been determined (Harwood, 2007; U.S. Environmental Protection Agency, 2005b; Wiggins et al., 2003). Comparisons among MST methods have been made in several studies (Griffith, Weisberg & McGee, 2003; Moore et al., 2005; Stoeckel & Harwood, 2007). Among the major drawbacks of library-dependent methods was their tendency to false-positive results (detection of contamination from a source when not actually present).

25. **Library-independent** MST methods are less subject to many of the concerns noted above, although careful method validation is still crucial (Griffith et al., 2003;

Moore et al., 2005; Stoeckel & Harwood, 2007). Library-independent methods generally rely on detection of a specific gene found in a microorganism that is unique to a certain host species (e.g. cattle) or group of hosts (e.g. ruminants). PCR is a generally used and widely accepted method to detect the source-specific microbe. In contrast with library-dependent methods, errors from library-independent methods tended more toward false-negative results, particularly in fecal samples from individual animals or humans (Griffith et al., 2003; Moore et al., 2005; Stoeckel et al., 2004). Sensitivity (the frequency of positive results when the contaminating source is present) and specificity (the frequency of negative results when the contaminating source is absent) are among the most important attributes of a useful MST test (Santo-Domingo & Sadowsky, 2007; Stoeckel & Harwood, 2007; U.S. Environmental Protection Agency, 2005b). Therefore, the library-independent methods of MST are reliable tools for fecal source determination provided that the methods are properly validated for sensitivity & specificity; such validation also provides an error rate for the assay. ***Specificity testing should focus on the non-target fecal sources that are most likely to affect water quality in a given area; it is not possible or necessary to test specificity against every animal species known to exist in the area.***

26. Absolute specificity is extremely difficult to achieve in any biologically-based assay, including those used for MST. Thus, "specificity" in the science of MST (and as a statistical concept) is a statistic that is calculated by determining the percent of true-negative results among all samples that should not contain the target or marker (Stoeckel and Harwood, 2007). The desired trait of "host-specificity" for MST markers has been frequently qualified in the literature in recognition of the difficulty in achieving absolute host specificity. For example, Stewart et al. used the term "...a predominantly host-specific microbe," (Stewart et al., 2003)and Santo Domingo et al. wrote that "...the *level* of host specificity is important in complex watershed..." (italics added) (Santo Domingo et al., 2007). Kildare et al. referred to their newly-developed qPCR assays as human-specific, dog-specific, and cow-specific even though limited cross-reactivity with other host species was observed for each of them (Kildare et al., 2007). Furthermore, in that study, results from previously published "host-specific" qPCR assays (Layton et al., 2006; Seurinck et al., 2005)also showed limited cross-reactivity with non-target fecal

samples. The human-specific HF183 assay developed by Bernhard and Field (2000) displayed ~95% specificity in Europe, but was still deemed "reliable" (Gawler et al., 2007). The number and type of samples that should be tested for specificity is dependent upon the question being asked in the study, as outlined by this quote from the U.S. EPA Microbial Source Tracking Guide Document (U.S. Environmental Protection Agency, 2005b)(pg 55):

> "Although there is currently no consensus, specificity values below 80% percent reflect questionable discriminatory power. Managers should use data with caution and may need to consider data from an alternative MST approach. Specificity control standards should be prepared at concentrations easily detected by the respective MST method and should consist of a pool of fecal samples acquired from animal sources in the same geographic context as water samples. The minimum number of individual animal fecal samples will be dependent on the complexity of the watershed system (see Chapter 4). Currently, there is no agreement on how to calculate this number. A conservative estimate might be a minimum of ten individuals per animal source. Because specificity control standards are generated for each watershed, specificity must be established for each geographic location tested. "

27. We developed a library-independent, PCR-based assay for detecting fecal-contaminated poultry litter in the IRW. In so doing, we followed the nearly decade-long precedent established by Bernhard and Field (2000), which has been the model for the development of many other MST assays e.g. (Dick et al., 2005; Kildare et al., 2007; Layton et al., 2006; Lu, Santo Domingo & Shanks, 2007; Okabe et al., 2007; Shanks et al., 2008; Shanks et al., 2007).

28. The methodology used to develop the poultry litter biomarker assay (hereafter referred to as the PLB) is described in detail in Section V of my expert report. An abbreviated synopsis is presented below. Method development began with identification of candidate bacteria that were widespread (prevalent) in fecal-contaminated poultry litter and also made up a substantial proportion (high concentration) of the bacterial population in the litter. Fecal-contaminated poultry litter rather than feces was used to develop the PLB to ensure that the MST target could survive deposition on poultry litter and subsequent spreading on fields. The method development can be divided into the

following stages: (I) target identification; (II) PCR development and validation of target sequence; and (III) quantification; (IV) further validation.

29. Target identification. The preliminary screening searched for bacterial DNA sequences that were represented in fecal-contaminated turkey and chicken litter, as well as in soils impacted by land application of poultry litter. DNA from three different groups was analyzed: *E. coli, Bacteroidales* (a bacterial family to which the genus *Bacteroides* belongs) and total bacteria. The 16S rRNA genes of each bacterial group were amplified by PCR, and terminal restriction fragment length polymorphism (TRFLP) was used to create DNA fragments that allowed identification of potential targets. No *Bacteroidales* fragments were represented in all litter and soil samples, therefore analysis of this group was not carried forward. Cloning and DNA sequencing of potential targets from the *E. coli* and total bacterial DNA pools was carried out to determine the precise sequence of each gene fragment. Each DNA sequence was compared to the worldwide NCBI (National Center for Biotechnology Information) database, which is a worldwide, centralized repository for DNA sequences. The DNA sequence comparison was used to screen out (discard) non-useful targets that had been identified in habitats or animals/humans other than the gastrointestinal tracts or feces of poultry. The DNA sequence screening process yielded four sequences that were ubiquitous in poultry litter and contaminated soil, and also contained unique sequences that allowed development of target-specific PCR primers. Three of these sequences were from the total bacteria DNA pool and one was from the *E. coli* DNA pool. The sequence derived from the *E. coli* pool was identified as the closely-related bacterium *Pantoea ananatis.*

30. PCR and validation of target sequence. A PCR assay was developed for each of the four potential targets described above. To increase the sensitivity of detection, a nested PCR approach was employed in which DNA was first amplified using universal bacterial primers (or all-*E. coli* primers) followed by amplification of an internal fragment with the target primers. Assay sensitivity was tested against composite poultry litter samples and against soil samples on which poultry litter had been land-applied. Specificity of the assays was tested against fecal samples from beef and dairy cattle, swine, ducks, geese, and human sewage. The collection and handling of these fecal

samples is detailed in Dr. Olsen's report (Olsen, 2008), but a brief description of the makeup of these samples is below.

31. Nontarget fecal samples (from animals other than poultry and human sewage) for specificity testing were collected as composites from groups of individuals. Independent duplicate samples were collected in all cases. Beef cattle fecal samples were collected from ten grazing fields. Two independent duplicate samples were collected for each field, and each duplicate consisted of feces from ten scats (feces from ten scats = 1 composite). A total of 200 beef cattle scats were tested in this phase. Duck (5 composites) and goose (5 composites) fecal samples were collected in the same fashion, consisting of composites from ten individual scats, and independent duplicates were collected for each area. For ducks, three landing areas inside the watershed and two outside the watershed were sampled, while for geese, two landing areas inside and three landing areas outside the watershed were sampled. Composite samples of fecal slurries were collected from two swine facilities (2 duplicate samples/facility) and three dairy cattle farms, human residential septic cleanout tanks (3 samples) and influent of three separate municipal wastewater treatment plants (3 samples).

32. The PCR assay with greatest sensitivity (consistently able to detect the target in contaminated samples and specificity (lack of detection in non-target samples) was produced by primer set LA35, which targets a 16S rRNA gene fragment of 571 base pairs that is 98% identical to the DNA of *Brevibacterium avium.* The sequence was detected in all litter samples, and in eight of ten contaminated soil samples. Among the non-target fecal samples, it was only detected in one composite goose and one composite duck sample, each of which was collected outside the IRW. Furthermore, the PLB was detected in only one of two duplicate samples from the cross-reactive duck and goose fecal composite, showing that it was present at very low concentration in these samples.

33. Quantification. Conventional PCR detects only the presence or absence of a target DNA sequence; it provides no quantitative information. Quantitative PCR (qPCR) has the added advantage of assessing the magnitude of the signal. Another advantage of qPCR is that it can provide an additional dimension of specificity, either by using an internal probe or by employing a melting curve. Again, we take advantage of the essential

biochemistry of DNA in that the double-stranded molecule will "melt" (dissociate into two individual strands) at a particular temperature. The melt curve of a given DNA sequence can be used to discriminate between it and nearly-identical sequences (Lin et al., 2008; Price et al., 2007). The instruments (thermocyclers) employed for qPCR can be programmed to raise the temperature in very regular increments to generate a highly reproducible melt curve.

34. Quantitative PCR. A qPCR assay was developed for the PLB using the LA35 primer set and Sybr green chemistry. The assay is quantitative, with a correlation coefficient of 0.9993; however, it cannot detect DNA concentrations as low as the ultra-sensitive nested PCR described above. The advantage of its quantitative ability outweighs the higher limit of detection, since it can still detect the biomarker in environmental water samples. A number of fecal samples were re-tested by qPCR for specificity, including the goose and duck duplicate that were each found to be positive by the ultra-sensitive nested PCR. Each of these samples was below detection limit, or negative by qPCR, including the duck and goose sample that were positive by conventional nested PCR. Seven newly-collected beef cattle samples (Camp Dresser & McKee (CDM), 2008) were assayed and three uncontaminated (clean) poultry litter samples were tested by qPCR. Each of these control (clean poultry litter) and non-target samples were negative, confirming the specificity of the PLB qPCR assay.

35. The concentration of fecal indicator bacteria in used poultry litter was compared to the concentration of the PLB to establish the relationship between the indicator organisms of fecal contamination and the poultry-specific marker. Enterococci concentrations were strongly and very significantly correlated with the PLB ($r^2 = 0.7471$; $P = 0.013$) (Figure 4 to my expert report), and *E. coli* concentrations also had a positive relationship with PLB concentration ($r^2 = 0.3946$; $P = 0.052$). The correlation of enterococci with the PLB remains when the 3 data points at the upper limit of the method detection (>120,000) are deleted from the data set. The FAC 01 sample that resembled soil was collected from a barn that had been largely cleaned out, thus it is not surprising that there is a low, but detectable residual level of enterococci and PLB in the sample.

36. The qPCR assay for the PLB was field-tested on litter, soil and water samples, including edge-of-field, surface water and ground water samples. A total of ten soiled litter samples, 187 water samples and 40 soil samples were tested. Three of the water samples were collected outside of the IRW where used poultry litter is not land-applied; therefore they represent reference water samples which should not contain the PLB. In fact, the PLB in each of these samples was not detected in the negative control (reference) samples. All contaminated litter samples contained very high concentrations of the PLB, ranging from $2.2X10^7$ - $2.5X10^9$ (tens of millions to billions) gene copies/g. The PLB was at high enough concentration to be quantified by qPCR in 34 water samples, including 16 edge-of-field samples, one groundwater sample (56287-7-13-06) and one spring sample (LAL15SP2-7-11-06). Six soil samples had quantifiable levels of the PLB, with the greatest at 3.8×10^6 gene copies/ml. .

37. When the PLB concentration was below detection limit in the qPCR assay, the nested version of this assay (which is presence-absence, rather than quantitative) was used to determine if lower levels of the PLB were present. The identity and purity of the PCR product was always checked by conducting a melting curve analysis. This nested Sybr green procedure allowed detection of the PLB in many samples in which the PLB was at too low a concentration to quantify. Of 40 total soil samples collected from fields that received land-applied poultry litter, 38 had detectable levels of the PLB. Of 187 water samples (including 3 reference unimpacted samples) 99 had PLB levels below the detection limit, but 88 water samples had detectable levels of the PLB. A total of 3 spring or groundwater samples had detectable or quantifiable concentrations of the PLB, demonstrating transport of poultry waste in the subsurface.

III. Additional knowledge about the poultry litter biomarker has been gained since the production of my Expert Report.

38. Analysis of additional samples from across the United States has been conducted based on reviewer's suggestions. A total of 116 non-target samples have now been analyzed by qPCR for the PLB from Colorado, Idaho, Florida, Minnesota, Missouri, Ohio and West Virginia. Eight of these samples [one duck, three goose, and four

wastewater treatment plant influent (raw sewage)] samples for specificity of 93%. When composite samples are taken into account, the 116 samples represent 394 fecal samples.

39. Seven additional poultry litter samples from Georgia were analyzed, and all were positive, as were four of five composite slurry samples from a Florida layer facility. Of 18 fecal samples from chickens (Georgia, Florida, Minnesota and Utah) twelve were positive; and one of two turkey fecal samples was positive. All told, 42 poultry samples (feces, slurry or litter) were analyzed and 32 were positive for the PLB (76% sensitivity).

40. Dr. Michael Sadowsky at the University of Minnesota independently replicated the results produced by North Wind, Inc. (Sadowsky, 2009). Dr. Sadowsky was sent a positive control and DNA extracts that were coded so that their source was unrecognizable. These samples had previously been analyzed by North Wind and consisted of 13 used poultry litter samples from four different facilities, 31 composite, non-target fecal samples (10 beef cattle, 3 dairy cattle, 2 swine, 5 geese, 5 duck, and 6 raw human sewage), four soil samples from fields on which poultry litter had been spread, and five edge of field runoff water samples. The lab also received DNA extracted from 11 environmental water samples that may have been impacted by land application of poultry litter, and three reference samples collected from areas with no known land application. The qPCR PLB assay was optimized for the thermocycler instrument at his lab and the samples were analyzed. Sadowsky's results corroborated the North Wind results; he found high concentrations of the PLB in each of the used poultry litter samples and obtained negative results for all non-target samples. His lab also detected the PLB in two soil samples (50%) and in three edge of field samples (60%). Two of the 11 water samples were positive for the PLB, while all three reference (unimpacted) samples were negative.

41. Peer review of this work was conducted prior to the addition of the samples from other geographic areas and Dr. Sadowsky's replication of the method. The manuscript was submitted to Applied and Environmental Microbiology (AEM) twice. As pointed out by other experts on this case, peer review is not a perfect process and reviewers can make mistakes; however, the process generally produces an improved finished work. The paper was extensively edited for the second submission, as the initial

version was overly complex. The editor clearly stated the reviewers' greatest concern regarding the second version: "One of the most serious concerns is the potential for application of the method to other geographic regions, as other studies have shown that these biomarkers lose specificity when tests are conducted using samples from a broader geographic field regardless of the assurance made that these primers may have a broader application. Other concerns are over the lack of necessary controls and the lack of appropriate statistical analyses to support your conclusions. For these reasons, and the reasons in the attached reviews, I am unable to accept your manuscript for publication."

42. The broader geographic applicability of the PLB has been addressed by adding the samples mentioned above. One reviewer had reservations about the use of composite samples for sensitivity testing due to the perception that it could inflate the true-positive rate. In fact, it was not possible to obtain individual fecal samples from poultry in the IRW. We did succeed in obtaining some poultry fecal samples from other states, and found that 7 of 14 individual samples and 89% of composite samples were positive; this information will be included in the revised publication. It is important to keep in mind the purpose of MST testing; one is not concerned with the waste of one animal, but with multiple animals that contribute significant amounts of fecal pollution to the watershed. Our MST paper using human polyomaviruses as a qPCR target was very recently published in Applied and Environmental Microbiology; it has demonstrated 100% sensitivity toward composite (human sewage) samples, but only 23% sensitivity toward individual samples (McQuaig et al., 2009). The review has only one comment about the statistics: "Finally, the regression data presented in Figure 4 is meaningless. R^2 is a guide to the "goodness-of-fit" of the data to the regression line. It does not indicate whether there is an association between the variables that is statistically significant. In order to demonstrate this, the authors should have performed an analysis of variance of the regression (a simple procedure given modern statistics packages)." While it is true that we should have added a P-value to the manuscript to show statistical significance, that is an omission that is very easily remedied.

43. In terms of sensitivity, specificity, and extensive validation, the poultry litter biomarker work meets or exceeds the standards established in the literature. It has known error rates in terms of sensitivity (11% with respect to the type of composite samples one

would expect to detect in water bodies) and specificity (7%). It is worth noting that the probability of a false-negative (failure to detect the target when contamination from poultry is present) is greater than the probability of a false-positive (detection of the target when contamination from poultry is absent). To my knowledge, it is the only MST methods that will be validated by an independent laboratory using blind samples when it is first published. A final note on specificity follows, quoted from (Field et al., 2003):

"For any method of fecal source tracking, there will be an associated rate of false positives. As long as the rate is relatively low, and measurable, it does not negate the usefulness of the method. Careful studies could establish the expected rate of false positives for each of these methods."

IV. CONCLUSIONS

44. Soiled poultry litter contains high levels of indicator bacteria and human pathogens, such as *Campylobacter* and *Salmonella*. These pathogens can be transmitted to humans via the waterborne route, and children are among the most vulnerable to infection and serious illness. Poultry litter is disposed of in the IRW by land application, which is a practice that is known to impact nearby water bodies with microbial and nutrient contamination. Elevated levels of fecal indicator bacteria are used to monitor human health risk from recreational water use, and many tributaries and segments of the Illinois River do not meet the U.S. EPA's ambient water quality criteria. Testing of poultry litter, soils upon which poultry litter has been applied, and edge-of-field samples collected from ditches during runoff conditions all show high levels of fecal indicator bacteria, some of which approach the levels expected in raw sewage. When these bacteria reach the extensive network of IRW tributaries, they become dominant contributors to the fecal indicator bacteria loads that impair the use of the Illinois River and its tributaries as recreational waters. Sampling of IRW surface water, groundwater, soil and sediments has revealed a unique chemical and bacterial signature that indicates contamination by poultry; and this signature is not present in areas that are remote from poultry operations. The finding that a poultry litter-specific biomarker (PLB) is found in all environmental compartments tested in the IRW, from soil samples to edge-of-field samples to surface water and groundwater, firmly links a dominant portion of the indicator bacteria

contamination to poultry waste. Thus, the disposal of poultry waste by land application in the IRW presents a substantial, serious and immediate threat to human health.

45. The theory of PCR and its use to detect specific microorganisms in the environment is not novel; in fact, it is grounded in two decades of scientific study. Likewise, the theory behind microbial source tracking, that specific microorganisms are associated with the feces of certain animals, and can be used to trace fecal contamination in the environment, is supported by over two decades of research. The poultry litter biomarker has a known error rate and has been validated in a manner that is consistent with the most recent and rigorous scientific literature. The PLB alone is not the basis for my opinion on water quality and human health risk in the IRW; rather, the weight of evidence from all the avenues presented must be taken into account to arrive at an informed opinion.

46. Please note that my opinions in this matter are my own, and do not reflect an official view of the University of South Florida.

I declare under penalty of perjury, under the laws of the United States of America, that the foregoing is true and correct.

Executed on the 26th day of May, 2009.

Valerie J. Harwood

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I declare under penalty of perjury, under the laws of the United States of America, that the foregoing is true and correct.

Executed on the 26th day of May, 2009.

Valerie J. Harwood

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